Effect of a Herbal Formula Consisting of Leech, Dahuang and Chinese Cassia Bark on Diet-induced Atherosclerosis in Rabbits

Heqing Huang[1] PhD
Peiqin Liu[1] PhD
Weihua Liu[1] MSc
Sha Tao[2] PhD
Zhi-Wei Zhou[3] BSc
Ziqing Hei[4] MD
Chun Guang Li[5] PhD
Charlie Changli Xue[5] PhD
Min Huang*[1] PhD
Shu-Feng Zhou*[6] PhD

1. School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, China
2. Sun Yat-sen College of Medical Sciences, Sun Yat-sen University, Guangzhou, China
3. College of Bioengineering, Nanchang University, Nanchang, China
4. First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China
5. The Chinese Medicine Research Group, Division of Chinese Medicine, RMIT University, Melbourne, Australia
6. Division of Pharmacy, School of Life Sciences, Queensland University of Technology, Brisbane, Australia

ABSTRACT

Atherosclerosis is a common condition with slow build-up of plaque on the inside wall of arteries. This study was undertaken to investigate whether a herbal formula consisting of three traditional Chinese medicines, including leech (*Shuizhi*, *Whitmania pigra* Whitman), *Dahuang* (medicinal rhubarb, *Rheum palmatum* L., Polygonaceae), and Chinese cassia bark (*Guipi*, *Cinnamomum cassia* Blume, Lauraceae) had beneficial effects on diet-induced atherosclerosis in rabbits. This herbal formula has been traditionally used to treat symptoms presented in stroke and ischaemic heart disease by Chinese doctors for more than 2000 years. Experimental atherosclerosis was established by feeding New Zealand white rabbits (*Oryctolagus cuniculus* L.) with a high-cholesterol diet for 10 weeks. The study demonstrated that the high-cholesterol diet resulted in significantly thickened aortic intima, enhanced intima area (with a total plaque area of 46.87%), marked apoptosis in plaques, elevated plasma levels of total cholesterol, triglyceride, high density lipoprotein (HDL-C), low density lipoprotein (LDL) and malonyldialdehyde (MDA), and significantly increased aortal ceramide content and sphingomyelinase (SMase) activity at the end of the 10-week period. Treatment of the atheromatous rabbits with the compound herbal formula at 1.5 g/kg significantly decreased the area of aortal plaque (to 28.62%) and apoptosis, and brought down the increased plasma MDA levels, aortal SMase activity and ceramide content to normal levels. These results suggest that the compound herbal formula has inhibitory effects on the development of atheromatous plaques in rabbits, probably through anti-oxidative effects and inhibition of apoptosis and ceramide production.

KEYWORDS atherosclerosis, apoptosis, ceramide, leech, *dahuang*, *guipi*, *Whitmania pigra*, *Cinnamomum cassia*, *Rheum palmatum*.

* Correspondent authors; e-mail: s4.zhou@qut.edu.au or huangmin@mail.sysu.edu.cn

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Introduction

Diseases caused by atherosclerosis are the leading cause of illness and death in many countries.[1-3] Atherosclerosis is characterised by clogging, narrowing and hardening of medium-sized and large arteries, due to slow build-up of fatty deposits (plaques) on the inside walls of the arteries; these plaques are made up of fat, cholesterol, calcium, and other substances found in the blood.[2,3] Atherosclerosis can cause serious complications, including stroke, heart attack, eye problems and nephropathy.[1,4] The risk factors for atherosclerosis include high blood pressure, smoking, diabetes, obesity, hyperhomocysteinemia, inflammation and hypercholesterolemia.[3,4] High blood cholesterol can modify the biochemical properties of blood components and arterial intima, promoting the progression of atherosclerosis.[2] In addition, increased oxidative stress within the vasculature, manifested by the accumulation of oxidised lipids and proteins, as well as the formation of reactive oxygen species, is commonly considered to play a key role in the initiation and progression of atherogenesis.[5] Sphingomyelinases (SMases) represent central elements of the so-called sphingomyelin/ceramide signalling pathway.[6,7] They play an important role in induction of cell proliferation, apoptosis, and cell activation, which are considered key events in atherogenesis. Stimulation of SMase activity produces ceramide, which has been shown to play an important role in oxidative, stress-mediated vascular endothelial cell apoptosis, which is involved in the development and progression of atherosclerosis.[8]

To help slow or reverse atherosclerosis, various medicines are needed to lower blood cholesterol (e.g. statins) and blood pressure (antihypertensives), and prevent clot formation, as well as blockade of blood flow (anticoagulants and antiplatelet agents).[9-11] Recently, novel therapeutic targets for atherosclerosis have been implicated, including those mediators and signalling molecules associated with angiogenesis and inflammation.[10,11] Despite the wide use of synthetic drugs, a number of herbal remedies are also used by many patients to lower cholesterol and treat atherosclerosis because of low cost, cultural tradition and wide availability.[12] Some herbal medicines, including *Danshen* (*Salvia miltiorrhiza* Bunge, Lamiaceae), *Yunzhi* (*Coriolus versicolor* (L. ex Fr.) Quél., Poriceae), and *Amirkabiria odoratissima* Mozaffarian (Umbelliferae), have been shown to have inhibitory effects on atherosclerosis progression in animal studies.[13-19] However, there is scant knowledge on their efficacy, mechanism of action and toxicity.

The herbal formula consisting of leech (*Shuizhi*, *Whitmania pigra* Whitman), *Dahuang* (medicinal rhubarb, *Rheum palmatum* L., Polygonaceae) and Chinese cassia bark (*Guipi*, *Cinnamomum cassia* Blume, Lauraceae) has been traditionally used to treat stroke and acute and chronic heart disorders by Chinese doctors for more than 2000 years.[20] The therapeutic use of leeches and *Dahuang* for salving ischaemic tissues in Oriental medicine dates back to 50 BC.[20] *Dahuang* as used in this formula is medicinal rhubarb (*Rheum palmatum* L., Polygonaceae), which is listed in the Chinese Pharmacopoeia. The Pharmacopoeia also lists *Rheum tanguticum* Maxim. ex Balf. and *Rheum officinale* Baill. under *Dahuang*. Leech (*Shuizhi*, *Whitmania pigra* Whitman, listed in the Chinese Pharmacopeia) demonstrates wide pharmacological effects, such as anti-platelet agglomeration and the decrease of blood lipids. It is frequently prescribed by traditional Chinese medicine doctors due to its conspicuous therapeutic effects, such as removing blood stasis and promoting blood circulation. In the clinic, it is widely used to treat cerebrovascular and other diseases. Active protein and amino acids are the major components of leeches. In particular, the leech saliva contains a peptide called hirudin, which is a direct thrombin inhibitor and a highly effective anticoagulant.[21] This study was undertaken to investigate whether the herbal formula of leech, *Dahuang* and *Guipi* had inhibitory effects on the atherosclerotic lesions in rabbits and the possible underlying biochemical mechanism for such effects.

Methods

CHEMICALS AND REAGENTS

2-Thiobarbituric acid and ceramide were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). Ultrapure water was prepared using a Milli-Q purification system (Barnstead International Inc., Dubuque, IO). Other chemicals and reagents were of analytical grade as appropriate. The extracts for the formula, which consisted of three traditional Chinese medicines – dried leeches, rhubarb, and Chinese cassia bark – were obtained in powder form from Guangdong Yifang 1st Pharmaceutical Factory (Guangzhou, China) and were produced to the standard of Good Manufacturing Practice. The ratio of the three traditional medicines in the mixture was 3:2:1. All these traditional medicines are registered in the Chinese Pharmacopoeia 2005 (www.sfda.gov.cn).

ANIMALS

Healthy male New Zealand rabbits (*Oryctolagus cuniculus* L.) (weight = 1.8 ± 0.3 kg, age = 5–6 months) were purchased from the Experimental Animal Center of Sun Yat-sen University, Guangzhou, China. The study proposal was approved by the Research Ethics Committee of Sun Yat-sen University. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.[22]

DIET-INDUCED ATHEROSCLEROSIS AND DRUG ADMINISTRATION

Male New Zealand rabbits (n = 8 in each group) were randomly divided into three groups: Group I (the blank control, fed with

standard rabbit chow (Purina Mills Inc., St Louis, MO)); Group II (treated with the control vehicle only, fed with standard rabbit chow containing 1% cholesterol); and Group III (treated with the herbal formula at 1.5 g/kg, fed with standard rabbit chow containing 1% cholesterol). The control vehicle contained powdered starch only. The animals were kept in a room under controlled temperature (22 ± 1°C), with an automatic day–night rhythm (12-hour cycle); they were housed in individual cages. The animals were fed fresh diets daily with free water access and their body weights were monitored regularly. The feeding amounts for each rabbit were limited to 135–150 g per day and the herbal mixtures were mixed in well with the feed. The dosage of the dried compound herbal formula was 1.5 g/kg body weight per day. This dosage was calculated based on the clinical dosage, with mean rabbit body weight about 1.8 kg. After 10 weeks of the experiments, the rabbits were anaesthetised with pentobarbital sodium at 50 mg/kg body weight by intraperitoneal injection. Blood was collected from the heart of each animal into a clear tube treated with heparin. Plasma was obtained by centrifugation at 1500 *g* for 8 min at 4°C. The animals were then sacrificed with the method of air embolism as described previously (a bolus of air at ~15 mL/kg in 5 s through the femoral vein).[23] Thoracic aortas were opened longitudinally and the inner surface was traced onto graph paper, with atheromatous plaques delineated.

MICROSCOPIC DETERMINATION OF PLAQUE AREA IN AORTAS

The numbers of small squares of the inner surface of aortas and areas surrounded by lines were counted using the National Institutes of Health plaque counting method.[24] Sections of the aortic sinus region were examined using light microscopy at 40× magnification with an Olympus BX60 optical microscope (Olympus Optical Co., Hamburg, Germany), and the cross-sectional area of lipid depositions was quantified using image-analysis software (Image ProPlus-4, Scitech).

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE-MEDIATED BIOTINYLATED UTP NICK END LABELLING (TUNEL) ASSAY

The TUNEL assay was performed in formalin-fixed and paraffin-embedded rabbit aortal tissue slides by using an apoptosis detection kit (DeadEnd Fluorometric TUNEL System, Promega, Madison, WI) according to the manufacturer's instructions. This system detects the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the terminal deoxynucleotidyl transferase (TdT). The fluorescein-12-dUTP-labelled DNA can then be visualised directly by fluorescence microscopy. An apoptosis index was obtained by counting the TUNEL positive cells. Results are means from three individual slides of the aortal tissue collected from at least six animals per experiment by counting 200 cells each slide.

MEASUREMENT OF PLASMA LIPIDS

The rabbit plasma levels of total cholesterol (TC), triglyceride (TG), high density lipoprotein-C (HDL-C) and low density lipoprotein (LDL) were detected by the enzyme method or one-step method as described previously using the Dimension RxL Chemistry Analyser (Dade Behring Diagnostics, Sydney, Australia) and relevant commercial kits (Boehringer Mannheim GmbH).[25] Lipoproteins were separated by a single density gradient ultracentrifugation for 18 hours at 21°C,[26] using 1 mL of rabbit plasma. The density gradient solution contained 0.25 mM EDTA and 0.1 mM butylated hydroxytoluene. Plasma lipids were extracted using 2.5 mL chloroform/methanol (2:1, v/v), vortex-mixed, and centrifuged at 1000 *g* for 15 min.[27] The lower phase of the Folch extract was evaporated under nitrogen and the lipids were then analysed. Cholesterol and triglyceride concentrations in plasma were expressed as millimoles per liter (mmol/L). Plasma protein concentrations were determined using the Lowry method.[28]

DETERMINATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY AND MALONYLDIALDEHYDE (MDA)

Superoxide dismutase (SOD) – one of the most important anti-oxidative enzymes – catalyses the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen. Plasma SOD activity was detected using a commercial kit SOD-525 (Oxis International Inc., Foster City, CA) by testing the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene (BXT-01050) in aqueous alkaline solution (pH 8.8) at an absorbance wavelength of 525 nm.[29] Briefly, the incubation was performed at pH 8.8 in 50 mM air-saturated 2-amino-2-methyl-1,3-propanediol buffer containing 3 mM boric acid and 0.1 mM diethylenetriaminepentaacetic acid at 37°C. The kinetic measurement of 525-nm absorbance was performed for 1 min upon addition of BXT-01050. The SOD activity was determined from the ratio of autoxidation rates measured in the presence and absence of rabbit plasma sample. One SOD activity unit was defined as the activity that doubles the autoxidation background.

Plasma content of MDA was measured by the 2-thiobarbituric acid method.[30] MDA is widely used as an indirect marker of lipid peroxidation. In brief, an aliquot of 1.0 mL rabbit plasma was pipetted into a screw-cap tube and mixed with 1.0 mL of 0.8% aqueous 2-thiobarbituric acid at final pH 0.9. The reaction mixture was then incubated for 30 min at 70°C with gentle agitation, cooled in an ice bath for 5 min, and tempered for 45 min at room temperature. Thereafter, the sample was analysed by third-derivative spectrophotometry (Shimadzu UV-160A) at 521.5 nm against a blank containing 1.0 mL of 5% aqueous trichloroacetic acid and 1.0 mL of 0.8% aqueous 2-thiobarbituric acid. Spectrophotometric conditions were set as follows: spectrum range, 400–650 nm; scan speed, 480 nm/min; and derivative difference setting ($\Delta\lambda$), 21 nm. The

2-thiobarbituric acid values (expressed as nmol of MDA per millilitre of plasma protein) were calculated on the basis of the calibration curve ($Y = 4.90 \times 10^{-3} + 9.01 \times 10^{-3} X$, where Y is peak height at 521.5 nm expressed in arbitrary units, as printed on the instrument chart, and X is the MDA concentration in nmol/mL of reaction mixture). All procedures were performed in attenuated light conditions.

DETERMINATION OF AORTAL SPHINGOMYELINASE (SMASE) ACTIVITY AND CERAMIDE CONTENT

The rabbit aortal samples were weighed and homogenised in 10 vol of chloroform-methanol 1:1 using a Polytron (Brinkmann Instruments Inc., Westbury, NY). The homogenate was filtered through a sintered glass funnel. The aortal SMase activity was determined using the radio-labelled enzyme tracing method as described previously.[31] In brief, 10 µL of collected rabbit aortal sample was added to the tubes followed by 50 mM Tris-HCl buffer (pH 9.0) containing 0.15 M NaCl, 2 mM EDTA, 10 mM taurocholate, 0.1 mM sphingomyelin, 2.0 µM [14C]-sphingomyelin (~20 000 dpm) to a final volume of 500 µL. After incubation at 37°C for 30 min, the reaction was terminated by adding 0.4 mL of chloroform-methanol (2:1, v/v) followed by centrifugation at 3000 *g* for 15 s. An aliquot (100 µL) of the upper phase containing the cleaved phosphocholine was analysed for radioactivity by liquid scintillation. The activity was expressed as nmol/h/mg in the upper phase after 30 min incubation.

The aortal ceramide content was determined using thin-layer chromatography as described previously.[32] Briefly, a total lipid extract was prepared according to Bligh and Dyer[27] and applied on Silica gel plates (60 F, 0.25 mm) for thin layer chromatography. The plates were developed by chloroform-methanol-25% ammonium hydroxide (65:25:4, v/v/v) and the lipid bands were visualised with iodine vapour. Only bands migrating with Rf values of known standards were quantified. Ceramide was developed with hexane-chloroform 1:1 followed by chloroform-methanol-acetic acid (91:2:3, v/v/v). Ceramide bands were visualised by charring, using a copper sulfatephosphoric acid reagent, and band density determined using a Bio-Rad 620 videodensitometer and 2-D Analyst software (Hercules, CA). Ceramide levels were normalised to phospholipid phosphate and are presented as means ±SD (pmol/nmol lipid phosphate) from at least three independent determinations.

STATISTICAL METHODS

Data are expressed as mean ±SD and were analysed using SPSS 10 (SPSS Inc., Chicago, IL). The statistical analysis to evaluate the differences in the mean parameters among the different groups was conducted by a one-way analysis of variance (ANOVA), followed by a post-hoc test (Dunnett's multiple comparison test). For multi-group comparison, the significance level (p) was set at 0.05 divided by the number of comparisons.

Results

Rabbits in all groups ate all of their chow during the period of the study, and showed gradual increases in body weight and serum lipid concentrations (Table 1). Statistically, no significant differences were seen in body weight among the three groups at the end of the study and the average body weights of the animals in Groups I, II, and III were 3.2 ± 0.4, 3.6 ± 0.5, and 2.9 ± 0.5 kg, respectively. The high-cholesterol diet resulted in significantly thickened aortic intima, and enhanced intima area, with significantly increased apoptotic cells (58%). The apoptotic cells (mainly macrophages and some muscle cells) had condensed chromatin along the nuclear membrane and shrunken nuclei.

Feeding of rabbits with a high-cholesterol diet for 10 weeks significantly increased plasma levels of TC, TG, HDL-C, and LDL (Table 1). The plasma TC level was increased from 1.66 ± 0.88 mmol/L in rabbits fed with standard chow to 38.23 ± 13.82 mmol/L when rabbits were fed with the high-cholesterol diet ($p < 0.001$). The plasma TG level was only moderately increased in rabbits fed with the high-cholesterol diet compared to rabbits fed with standard chow (1.31 ± 0.37 vs 0.86 ± 0.33 mmol/L, $p < 0.05$). Notably, the plasma LDL concentration was dramatically increased (205-fold) in rabbits fed with the high-cholesterol diet compared to rabbits fed with standard chow (0.23 ± 0.03 vs 47.25 ± 15.21 mmol/L, $p < 0.001$).

In contrast, the plasma level of SOD was significantly decreased in rabbits fed with the high-cholesterol diet compared to rabbits fed with standard chow (89.78 ± 23.73 vs 197.57 ± 28.87 U/mL, $p < 0.05$). Consistently, the plasma MDA level in rabbits fed with the high-cholesterol diet was significantly increased compared to rabbits fed with standard chow (17.29 ± 5.13 vs 9.28 ± 3.33 nmol/L, $p < 0.05$).

The high-cholesterol diet also significantly increased aortal SMase activity in rabbits compared to rabbits fed with standard chow (1.38 ± 0.35 vs 0.27 ± 0.02 nmol/h/mg tissue, $p < 0.01$). In addition, the aortal ceramide level in rabbits fed with the high-cholesterol diet was significantly increased compared to rabbits fed with standard chow (10.01 ± 2.04 vs 4.03 ± 0.73 pmol/nmol lipid phosphate, $p < 0.01$).

Treatment of the atheromatous rabbits with the compound herbal formula consisting of leech, *Dahuang* and *Guipi* at 1.5 g/kg for 10 weeks significantly decreased the area of aortal plaque (from 46.87% to 28.62%) and endothelial apoptosis (from 52% to 28%). However, the herbal formula only slightly

TABLE 1 A comparison of various parameters for rabbits fed on three different diets (mean ±SD of 8 animals per group)

Parameter	Group I (Standard diet)	Group II (High-fat diet)	Group III (High-fat diet + herbal formula)
Body weight	3.2 ± 0.4	3.6 ± 0.5[a]	2.9 ± 0.5[b]
Area of plaques (%)	0	46.87±15.56[a]	28.62 ± 13.29[a&b]
Apoptosis index (%)	2 ± 0	52 ± 18[a]	28 ±8[a&b]
Total cholesterol (mmol/L)	1.66 ± 0.88	38.23 ± 13.82[a]	33.48 ± 10.09[a]
Triglyceride (mmol/L)	0.86 ± 0.33	1.31 ± 0.37[a]	1.37± 0.34[a]
HDL (mmol/L)	0.60 ± 0.25	1.78 ± 0.43[a]	1.66 ± 0.42[a]
LDL (mmol/L)	0.23 ± 0.03	47.25 ± 15.21[a]	50.67 ± 15.32[a]
SOD (U/mL)	197.57 ± 28.87	89.78 ± 23.73[a]	195.22 ± 21.61[b]
MDA (nmol/mL)	9.28 ± 3.33	17.29 ± 5.13[a]	11.96 ± 3.39[b]
SMase (nmol/h/mg)	0.27 ± 0.02	1.38 ± 0.35[a]	0.32 ± 0.06[b]
Ceramide (pmol/nmol lipid phosphate)	4.03 ± 0.73	10.01 ± 2.04[a]	3.85 ± 0.91[b]

Notes: [a&b] $p < 0.05$ (ANOVA).
[a] Group I vs Group II or III; [b] Group II vs Group III (with adjusted p values).

decreased plasma total cholesterol (from 38.23 to 33.48 mmol/L), and HDL-C (1.78 vs 1.66 mmol/L). The plasma LDL level was slightly increased (from 47.25 to 50.67 mmol/L). However, co-treatment of the herbal formula slightly increased the plasma triglyceride level (1.31 vs 1.37 mmol/L).

Treatment of the atheromatous rabbits with the compound herbal formula consisting of leech, *Dahuang* and *Guipi* at 1.5 g/kg for 10 weeks significantly brought down the increased plasma MDA level to largely normal levels (Table 1). The compound herbal formula also significantly increased plasma SOD activity (from 89.78 to 195.22 U/mL). Moreover, the herbal formula inhibited aortal SMase activity (from 1.38 to 0.32 nmol/h/mg tissue) and decreased aortal ceramide level (from 10.01 to 3.85 pmol/nmol lipid phosphate).

Discussion

The results demonstrated an anti-oxidative effect of the compound herbal formula, as indicated by increased SOD activity and reduced MDA levels. In the case of the hyperlipidaemia, the anti-oxidative capacity of the body decreases, excessive oxygen groups and free radicals are produced, and lipid oxidisation is promoted to produce a large amount of lipoperoxide and the degradation product, MDA.[3,33] The absence of suitable compensatory mechanisms from endogenous anti-oxidant systems causes a redox imbalance and leads to the activation of stress-sensitive intracellular signalling pathways. The increased production of reactive oxygen species can lead to damage of proteins, lipids, and DNA. LDL is the carrier of lipoperoxide which can be oxidisationally modified to form the ox-LDL.[3,33] Ox-LDL could induce the apoptosis of the vessel endothelial cells and affect the regeneration of these cells and the vessel endothelium-dependent relaxation. Ox-LDL is easily absorbed and phagocytised by the monocytes/macroghages which will be activated and release a number of mediators such as cytokines in the damaged intima, probably contributing to the pathogenesis of atherosclerosis.[2,3,8,33]

In the present study, we found that co-administration of the herbal formula reduced the apoptosis induced by a high-cholesterol diet in aortal plaque cells. The effects of leeches, *Dahuang* and *Guipi* and their active constituents on cell proliferation and apoptosis appear to be divergent. Some studies have shown that their components can induce apoptosis and inhibit the growth of a variety of tumour cell lines. For example, emodin isolated from *Dahuang* can induce multiple myeloma cell apoptosis through Janus-activated kinase 2 inhibition.[34,35]

As shown in Table 1, the development of diet-induced atherosclerosis was accompanied by an increased aortal SMase activity and ceramide, the second lipid messenger. The ceramide-mediated signal pathway is an important signal transduction system between the cell membrane and the nucleus.[36] Ceramide is formed from the sphingophospholipid in the membrane which was hydrolysed by the phospholipidase C. Ceramide can be degraded by ceramidases to sphingosine, and this, in turn, can be phosphorylated by sphingosine kinase to produce sphingosine-1-phosphate.[6] Both sphingosine and sphingosine-1-phosphate have been implicated in the regulation of cell proliferation and death.[6] Ceramide can activate a series of protein kinases and protein phosphatases of the 2 A family to initiate intracellular phosphorylation, induce the expression of a number of important proteins that regulate cellular apoptosis, senescence, proliferation or differentiation.[37] The ceramide signal pathway plays a key role in the development and the progression of atherosclerosis.[37] Decreased ceramide could contribute to the inhibition of apoptosis.

Treatment of the atheromatous rabbits with the compound herbal formula consisting of leech, *Dahuang* and *Guipi* at 1.5 g/kg for 10 weeks only slightly decreased total cholesterol and HDL levels and slightly increased LDL concentration. It appears that the herbal formula has a marked anti-oxidant effect in atheromatous rabbits, but its hypolipidaemic activity is minor to moderate.

Triglyceride is a higher risk factor than total cholesterol to arthrosclerosis-related coronary heart disease and stroke. However, the herbal treatment tended to increase serum triglyceride level from 1.31 to 1.37 mmol/L. The reason for this is unknown. The herbal formula may temporarily increase triglyceride synthesis and release from the liver when modifying fat acid metabolism.

In conclusion, the anti-oxidative capacity and inhibition of aortal cellular apoptosis and ceramide production by the compound herbal formula appear to be the major reason for the beneficial effects observed in this study. Further research is needed to explore the molecular mechanism underlying the beneficial effect of the compound herbal formula on atherosclerosis.

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